

STATISTICAL OPTIMIZATION OF CULTIVATION CONDITIONS FOR THE ENHANCED PRODUCTION OF BACOSIDES FROM *Bacopa monnieri* CELL SUSPENSION CULTURE

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STATISTICAL OPTIMIZATION OF CULTIVATION CONDITIONS FOR THE ENHANCED PRODUCTION OF BACOSIDES FROM *Bacopa monnieri* CELL SUSPENSION CULTURE

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under the supervision of

Dr. Nivedita Patra



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May 23, 2017

Supervisor's certificate

This is to certify that the work presented in the dissertation entitled *Statistical Optimization of Cultivation Conditions for the Enhanced Production of Bacosides from Bacopa monnieri Cell Suspension Culture* submitted by *Bishwanath Seth*, Roll Number 215BM2012, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology in Biotechnology*. Neither this thesis nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

Dr. Nivedita Patra
Assistant Professor

Dedication

I would like to dedicate the success of this research to Dr. Nivedita Patra who guided me throughout the project work. I would also like to dedicate to my parents, my friends and my lab mates for their help and constant motivation towards the completion of this project.

Bishwanath Seth

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May 24, 2017
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Declaration of originality

I, *Bishwanath Seth*, Roll Number 215BM2012 hereby declare that this dissertation entitled *Statistical Optimization of Cultivation Conditions for the Enhanced Production of Bacosides from Bacopa monnieri Cell Suspension Culture* presents my original work carried out as a postgraduate student of NIT Rourkela and, to the best of my knowledge contains no materials previously published or written by another person, nor any material presented me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in this dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections “Reference” or “Bibliography”. I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation

May 23, 2017

NIT Rourkela

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Abstract

Bacopa monnieri is commonly called as Brahmi belongs to the family Schrophulariaceae. It is found in wet, shady and marshy areas. This plant has high demand in commercial application due to its medicinal properties. It has been extensively explored by researcher due to the presence of bacoside; a memory enhancer used to treat memory disorder. Due to its high demand in commercial application the natural habitat of this plant depleted continuously and has been already entitled in the list of threatened species by International Union of conservation of nature and national resources. The best way to save this plant from being endangered is to utilize this plant by plant tissue culture to extract the important metabolites. Production of bacoside through plant tissue culture is very low. The aim of this research is to increase the yield of bacoside production through plant tissue culture. To increase the yield of bacoside a central composite design (CCD) was performed to optimized the media in suspension culture. Three factors such as inoculum size, sucrose concentration and phosphate concentration was optimized. The maximum predicted yield for biomass was found to be 3.65 g/L DW when the media contains 2 g/L inoculum, 30 g/L sucrose and 1.24 mM phosphate concentration. The maximum predicted yield for bacoside was 0.49 mg/g DW when the media contains 1.98 g/L inoculum, 41.92 g/L sucrose and 0.22 mM phosphate. Experimental validation of the model predicted optimized media was performed and the correlation between experimental and model predicted value was found to be 99 % for biomass and 94 % for bacoside. There was 2.23-fold improvement in biomass and 3.63-fold improvement in bacoside production in optimized media. The nutrient (sucrose, nitrate and phosphate) consumption by the cell biomass in the optimized media was also studied and it was observed an initial lag phase of three days followed by a log phase from day third to day sixth in which the rate of nutrient consumption was very high and finally a stationary phase at the end of the twelve days' study. Elicitation study was also performed to enhance the yield of bacoside in optimized media. Methyl Jasmonate at a concentration of 5 mg/L was used as elicitor on day four to elicited the production of bacoside. It was found that there is an increase in bacoside production; a 1.4-fold increase as compared to the control.

Keywords: *Bacopa monnieri*; Biomass; Bacoside; Central composite design; Elicitation

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
BAP	Benzyl Amino Purine
CuSO ₄	Copper Sulphate
DW	Dry weight
DCW	Dry cell weight
HPLC	High Performance Liquid Chromatography
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
Kn	Kinetin
MJ	Methyl Jasmonate
MS	Murashige Skoog
NAA	Napthalic Acetic Acid
NO ₃	Nitrate
SA	Salicylic Acid
TDZ	Thidiazuron

List of Symbols

g	Gram
L	Litre
μM	Micro molar
mM	Milli molar
%	Percentage
mg	Milligram
$^{\circ}\text{C}$	Degree Celsius
rpm	Revolution per minute

Chapter 1

Introduction

Bacopa monnieri is commonly Known as ‘Brahmi’ belongs to scrophulariaceae family. The other names include water hyssop, herb of grace, Indian pennywort, thyme leafed gratiola. It is a small, perennial, succulent creeping herb with fleshy leaves found all over the India in wet, shady and marshy areas (Satyavati et al., 1976). It is also found in Nepal, Srilanka, Taiwan, Vietnam, Florida state and other southern state of America.

It is an ancient medicinal plant used in Ayurveda medicine. In Ayurveda, *B. monnieri* has been classified under medharasayana. It has been prescribed for the promotion of memory, intelligence, and general performance. *B. monnieri* is one of the most important Indian medicinal plants evaluated on the basis of their medicinal importance, commercial value and potential for further research and development placed second in a priority list. The active principle constituents, reported in *Bacopa monnieri* are alkaloids; Brahmin, saponins, Herpestine, bacoside A, bacoside B, betulic acid, hersaponin, σ -sterol β -setosterol (Bose and Bose, 1931). The memory enhancing effects have been attributed to the presence of saponins, Bacoside A and Bacoside B. Bacoside is a natural memory booster acts on central nervous system (CNS) where it improves grasping power, memory, intellect and speech, and correct aberrations of emotions, mood and personality of an individual (Roodenrys et al., 2002). It is used for anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome and gastric ulcers (Shakoor, 1994). It has also anti-inflammatory, insanity, anticancer, analgesic, antioxidant and antipyretic activities (Pandiyana and Selvaraj, 2012). It is also used to treat hoarseness, blood purification and water retention. The juice of Brahmi leaves is used to cure diarrhea and bronchitis in case of children (Binita et al., 2005). *B. monnieri* has also the ability to phytoremediate toxic heavy metals like cadmium, mercury, chromium from aquatic bodies by absorbing and accumulating these metals in their shoots and roots (Ali et al., 2001; Shukla et al., 2007). Brahmi increases the neurotransmitter ‘serotonin’ present in brain which helps to increase relaxation (Rastogi and Kulshreshtha, 1999).

Bacoside A is the major chemical entity which shown to be responsible for the memory facilitating action (Rajani, 2008; Russo and Borrelli, 2005). It is a mixture of chemicals comprised of bacoside A3, bacoside-II, bacosaponin C and Jujubojenin isomer of bacosaponin C (Deepak et al., 2005). Bacoside is the principal active component present in

all parts of *B. monnieri* (Mathur et al., 2002) used to treat memory disorder. Current focus on bacoside is for the treatment of Alzheimer's disease. All the plant's derived drugs used for Alzheimer's disease are based on cholinergic hypothesis as these are the natural sources of Acetylcholinesterase inhibitors (Mukherjee et al., 2007; Murray et al., 2013). Bacoside act through inhibition of the enzyme acetylcholinesterase (AChE) and activation of the synthesis of Acetylcholine. Although recently several synthetic drugs have been introduced to treat learning and memory disorder, but their therapeutic effects are low and most of them have undesirable side effects, whereas plants derived drugs have no side effect or very low toxicity to the body. Bacoside improves the impulses transmission between neurons and repaired the damaged neurons by degenerating synapses which helps us to remember and learn information easily.

The natural habitat of this plant has been depleted due to the high demand of commercial application and it has been already entitled in threatened list by International Union for Conservation of Nature and National Resources (Tiwari et al., 2001). The alternative way to save a plant from being endangered is to produce in vitro plant by both differentiated cultures and de-differentiate culture and used of in vitro explant to produce metabolite in suspension culture as well as in callus culture is the best approach to save a plant from being endangered (Rajani, 2008). Propagation of the plant through seed is undesirable due to the low viability of seed and frequent death of seedling at the stage of two leaves. However, it is easy to propagate through stem cutting.

Production of bacoside through plant tissue culture is very low; 0.2 % and it's need to be improved (Tejavathi and Shailaja, 1999). To increase the yield of bacoside it is needed to be exploring the application of suitable elicitors. Elicitation is a complex process and there are many factors which influence the process such as concentration of elicitors, exposure time of elicitors, growth stage of the biomass at the time of elicitor addition and (Bourgaud et al., 2001; Namdeo, 2007; Zhao et al., 2005).

Chapter 2

Objectives

1. Response surface methodology to determine the optimum concentration of the selected factors (Inoculum size, Sucrose concentration and Phosphate concentration) in suspension culture for maximum biomass and bacoside production.
2. Experimental validation of the model predicted optimized media.
3. Yield enhancement strategy for maximum bacoside production by elicitation.

Chapter 3

Literature review

There are different materials and methods have been found in different research articles. There are variations in the surface sterilization and hormone supplements to the MS basal media. Different concentration of auxins and cytokinins are used for the callus induction. Different factors such as plant growth regulators, sugar concentration, pH has been optimized for callus culture. In case of HPLC it has been found that different mobile phase is used in different article. This review also includes the suspension culture and the statistical methods for media optimization such as Response surface methodology (RSM), nutrient consumption and elicitation study.

In one of the article it has been reported that for surface sterilization the explants were first rinsed in water for 5-10 minutes. Then the explants were soaked in an aqueous solution containing 0.2-0.5 % bavistin and 0.03 % streptomycin for 10 minutes. Then it was washed in sterile distilled water twice and treats with aqueous solution of savlon liquid. Then it was in sterile distilled water twice and finally treats with 0.01 % HgCl₂ for 1 minute and washed twice in sterile double distilled water before inoculation to the culture media (Vijayakumar et al., 2010).

In this article 0.1 % Tween-20 followed by immersion for 2-3 min in sodium hypochlorite (0.5%) and 1-2 min in mercuric chloride (0.5 %) solution has been used for surface sterilization. MS medium supplemented with 0.2 mg and 0.4 mg of 2,4-D and sucrose (30 g/L) for leaf explant; NAA (0.2 mg), BAP (0.1 mg) and sucrose (30 g/L); BAP (0.1 mg), KIN (0.2 mg) and sucrose (30 g/L) for nodal explant; NAA (0.1 mg), BAP (0.3 mg) and sucrose (30 g/L) for internode explants have been used for callus culture (Dharishini et al., 2014).

In this article it has been reported that highest callus growth was obtained in the treatment MS+2,4-D (2.0 mg/l) which recorded 15.20 g after 4 weeks and 35.60 g after 8 weeks of culture respectively. Auxins viz. IAA, IBA, NAA, 2,4-D in the concentration ranging from 0.2 mg/l to 5.0 mg/l, individually supplemented with MS medium has been used for callus induction. (Talukdar, 2014)

In this article it has been reported that MS medium supplemented with 4.0 mg L⁻¹ 2,4-D and 4.0 mg L⁻¹ NAA proved to be the most efficient hormone in promoting callus

development from the leaf explants with 42.0 % response and was the most favourable medium (Jeyakumar and Vivekanandan, 2015).

In this article it has been reported that MS medium supplemented with 3 % sucrose and plant growth regulators such as 2.5 mg/L BAP and 0.01 mg/L IAA gave the highest result (Jain et al., 2012).

In this article it has been reported that nodal explants were used to induce shoot buds in MS media supplemented with different concentration of 6-benzyl adenine 1-5 mg/L and it was observed that MS media supplemented with 3 mg/L 6-BA gave the best result; an average of 6.5 shoots bearing buds per node. It was sub cultured in MS media supplemented with 1mg/L Gibberellic acid which gave the best result; 114.2 buds per node (Behera et al., 2015).

In this article it has been reported that 0.05M Sodium Sulphate buffer at pH 2.3 and Acetonitrile (68.5: 31.5, v/v) at flow rate 1ml/min was used as mobile phase. C18 column was used for the separations. Column temperature was 30 °C. Detection was at 205 nm (Naik et al., 2010).

In another article it has been reported that Acetonitrile (A) and solution of potassium dihydrogen orthophosphate, Ortho-phosphoric acid and HPLC grade water (mili Q) were used as gradient for mobile phase at a flow rate of 1.5ml/min. C18 column was used for the analysis. Column temperature was 30 °C. Injected volume was 20micro litre and the detection was at 205 nm (TG et al., 2014).

In this article it has been reported that Mobile phase of 315 volume of Acetonitrile and 685 volumes of 0.72 % (w/v) anhydrous sodium sulphate at pH 2.3 (pH was adjusted by H₂SO₄) was used at a flow rate of 1ml/min. Injected volume was 20 µL. The elution program was run for 75 minutes and the detection was at 205 nm. (Dowell et al., 2015).

In one of the article it has been reported that Acetonitrile: water (48: 52, v/v) at a flow rate of 1ml/min was used as mobile phase. C₁₈ column was used for the separations. Column temperature was 30 °C. The injected volume for each separations was 10 µL. The run time was 30 minute for each separation. (Sharma et al., 2013a)

In this article gradient of Acetonitrile (A) and water 0.05 % (v/v) Orthophosphoric acid (B) at a flow rate of 1.5 ml has been used as mobile phase. C18 has been used for the

separation. The elution program was 0-25 minute from 30:70 (A: B) to 40:60 and 25-35 minute from 40:60 to 60:40. Detection was at 205 nm (Deepak et al., 2005).

In this article Acetonitrile: water (67: 33) at a flow rate of 1.6 ml/min has been used as mobile phase. C18 column has been used for the separation. Run time was for 30 minutes. Detection wavelength was between 200-300 nm (Mohana and Padma, 2016).

In this article it has been reported that for suspension culture were incubated at 110 rpm and the temperature was 25 ± 2 °C. Cells were harvested at an interval of 10 days. Same medium was used in suspension culture as in callus culture. The medium components were MS salt supplemented with NAA (1 g/L), Kinetin (0.5 g/L), Casein hydrolysate (1 g/L) and Sucrose (30 g/L) (Rahman et al., 2002).

In one of the article it has been reported that five factors such as MS salt, sucrose, casein hydrolysate, IAA and BAP were studied in Plackett-Burman study. Out of the five factors three factors, MS salt, sucrose and BAP were screened and used for the RSM. Full Ms salts, 5.68 % sucrose and 10.42 μ M BAP were the optimized parameters (Singh and Chaturvedi, 2012).

In this article it has been reported that three variables such as pH (5-7), sucrose concentration (0.5-4) and micro salt concentration (0.5-4) were optimized in second order D-optimal design. BAP (2.2 μ m/L), 2,4-D (4.52 μ m/L) and 2iP (4.29 μ m/L) were used as growth regulators. Maximum callus was obtained when pH=5.81, sucrose concentration=2.58 and macros salt concentration=0.5 (Sundaram et al., 2013).

In another article it has been reported that media component such as carbon source (glucose/carbon), nitrogen source ($\text{NO}_3^-/\text{NH}_4^+$ ratio) and K^+ ion ($\text{KNO}_3/\text{NaNO}_3$) were studied for optimization. Glucose was found to be best over sucrose. Nitrate gave the highest biomass and K^+ ion at 40:20 ratios gave the best result. Then the optimized media was used for the (Kumar et al., 2014).

In this article it has been reported that for the enhancement bacoside A production from hairy root culture Plackett-Burman design followed by response surface methodology was used to optimized the media. From this study it was observed an increase in yield of biomass; a two-fold increase from 6.8 g/L FW to 12.99 g/L FW and the increase in bacoside was from 10.2 mg/g DW to 16.44 mg/g DW when the media supplemented with 41.72 g/L Glucose, 6.12 g/L KNO_3 , 0.33 g/L KH_2PO_4 and 0.52 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Bansal et al., 2015).

In this article it has been reported that central composite rotatable experimental design (CCRD) a response surface methodology was used to optimize the media component for the production of beta-carotene in suspension culture from *Daucus carota*. Design expert trial v.6.0.10 trial was used for the experimental design. Sucrose, dihydrogen phosphate Ammonium sulphate and potassium nitrate were the media component used for optimization. The maximum production of beta-carotene in optimized media found to be 13.61 µg/g DW as compared to 9.63 µg/g in media before optimization. The optimum value of the medium components was sucrose 3.25 %, phosphate 0.97 mM and nitrate 53 mM (Hanchinal et al., 2008).

In this article it has been reported that three variables such as sucrose concentration, nitrogen concentration and concentration of plant growth regulator thidiazuron (TDZ) was optimized by central composite design (CCD) for shoot bud response and shoot bud induction from leaf explant of *Solanum melongena*. The bud response was found to be 95 % when concentration of sucrose, nitrogen and TDZ was 2.65 %, 4.34 g/L and 0.67 mg/L respectively. The maximum number of buds induced per leaf explants were 10 when the MS basal media was supplemented with sucrose, nitrogen and TDZ at a concentration of 2.36 %, 4.02 g/L and 1 mg/L respectively (Naveenchandra et al., 2011).

In this article it has been reported that central composite rotatable experimental design (CCRD) was used to optimized the media components to maximize the yield of capsaicin production. Ammonium nitrate, potassium nitrate and calcium chloride were the media components used for optimization. The maximum yield of the capsaicin was found to be 36.32 µg/g; a one-fold increase as compared to the media before optimization. Batch kinetic study of capsaicin production and the rate of nutrient (sucrose, nitrate and phosphate) was also studied. Initially there was a lag phase of three days and after that log phase was started up to eighteenth day where the production of capsaicin was maximum and stationary phase was observed up to the end of the study. It was found that the rate of sucrose utilization was rapidly increase as compared to nitrate and phosphate. The percentage of sucrose, nitrate and phosphate utilization was 93.4, 64.65 and 63.75 respectively. Maximum DCW and maximum capsaicin production was 52.53 g/L and 1.123 mg/L respectively (Sagwan et al., 2011).

In this report it has been reported the production of triterpenoids by the utilization of different substrate such as nitrate and phosphate was estimated by batch kinetic study. Study was for 16 days. From this study it was found that there was an initial lag phase of two days.

Nitrate consumption was slower as compared to phosphate and was present till the last day of cultivation. Maximum triterpenoids was produced when maltose was the carbon source 31.08 mg/L followed by sucrose 21.6 mg/L and glucose 10.69 mg/L (Srivastava et al., 2011).

In this article it has been reported that different concentration of Salicylic acid (50, 100 and 200 μ M) and Jasmonic acid (100, 250 and 500 μ M) was used as elicitors to increase the yield of hypercirin and pseudohypercirin in shoot culture from *Hypericum hirsutum* and *Hypericum maculatum*. The study was performed for a period of 21 days. From this study it was found that salicylic acid at a concentration of 50 μ M produced the maximum yield of hypercirin; a 7.98-fold increase and pseudohypercirin; a 13.58-fold increase as compared to the control in *H. hirsutum* and salicylic acid at 200 μ M concentration gave the highest yield of hypercirin; a 2.2-fold increase and pseudohypercirin; a 3.94-fold increase in *H. maculatum* (Coste et al., 2011).

In this article it has been reported that three abiotic elicitors named as Salicylic acid, Jasmonic acid and CuSO₄ was used to enhance the production of bacoside in shoot culture in liquid media. The study was performed for a period of 3, 6 and 9 days. The culture elicited with 45 g/L CuSO₄ was produced higher amount of bacoside; a 1.4-fold increase as compared to the control after 9 days of study (Sharma et al., 2015).

In one of the article it has been reported that different concentration of Methyl Jasmonate and Salicylic acid (25, 50, 75, 100 and 150 μ M) and in combination (25 μ M MJ+25 μ M SA, 25 μ M MJ+50 μ M SA, 50 μ M MJ+ 25 μ M SA and 50 μ M MJ+50 μ M SA) was used to enhance the production of bacoside in shoot cultures in liquid media. The study was for 1-4 weeks. From this study it was found that 50 μ M of MJ and SA individually produced two and three-fold higher amount of bacoside as compared to the control respectively. Both in combination of 25 μ M+25 μ M produced five-fold higher as compared to control (Largia et al., 2015).

In this article it has been reported that different concentration of Methyl Jasmonate (50, 100, 150 and 200 μ M) was used as elicitor to enhance the production of bacoside A, from shoot culture in liquid media. This experiment was done in triplicate for five weeks and every week treated shoot and control were sampled. From this study it was found that 50 μ M of Methyl Jasmonate produced the higher amount of bacoside; a 1.8-fold increase as compared to control (Sharma et al., 2013b).

Chapter 4

Materials and methods

4.1 Root induction from shoot explant of *Bacopa monnieri*

All the chemicals were purchased from HiMedia. Brahmi plant used for the research was ‘CIM-jagriti’ a high yielding variety brought from CSIR-CIMAP, Lucknow. Rooting of the shoot explant were done to produce in-vitro plant. Shoot explants 2 to 3 cm long were taken from the field grown plant. Explants were surface sterilized before inoculum to the media. First the explants were rinsed in running water for 30 minutes. Then the explants were treated with 0.5 % Bavistin for 5 minutes. Then it was rinsed with sterile distilled water twice. Then the explants were treated with 1 % Sodium Hypochlorite (NaOCl) for 5 minutes and finally rinsed with sterile distilled water twice. Then the explants were inoculated to the MS media supplemented with 1 % Agar, 3 % Sucrose and IAA at a concentration of 1 mg/L at pH 5.8 and kept in the plant growth chamber at 16 hr photoperiod.

4.2 Callus culture of leaf explants of *Bacopa monnieri*

Leaf explants were taken from the in-vitro plant. Explants were surface sterilized with 0.5% Bavistin and inoculated to the MS media supplemented with 1 % Agar, 3 % Sucrose and plant growth regulators such as BAP (0.1 mg/L) and NAA (0.5 mg/L) at pH 5.8 and kept inside plant growth chamber at 16 hr photoperiod.

4.3 Response surface methodology (RSM)

RSM was performed to determine the optimum concentration level of the significant factors. The significant factors were inoculum size, sucrose concentration and phosphate concentration. Central composite design (CCD) is most commonly used surfaced design experiment was used to determine the optimum concentration of the screened factors (Singh and Chaturvedi, 2012).

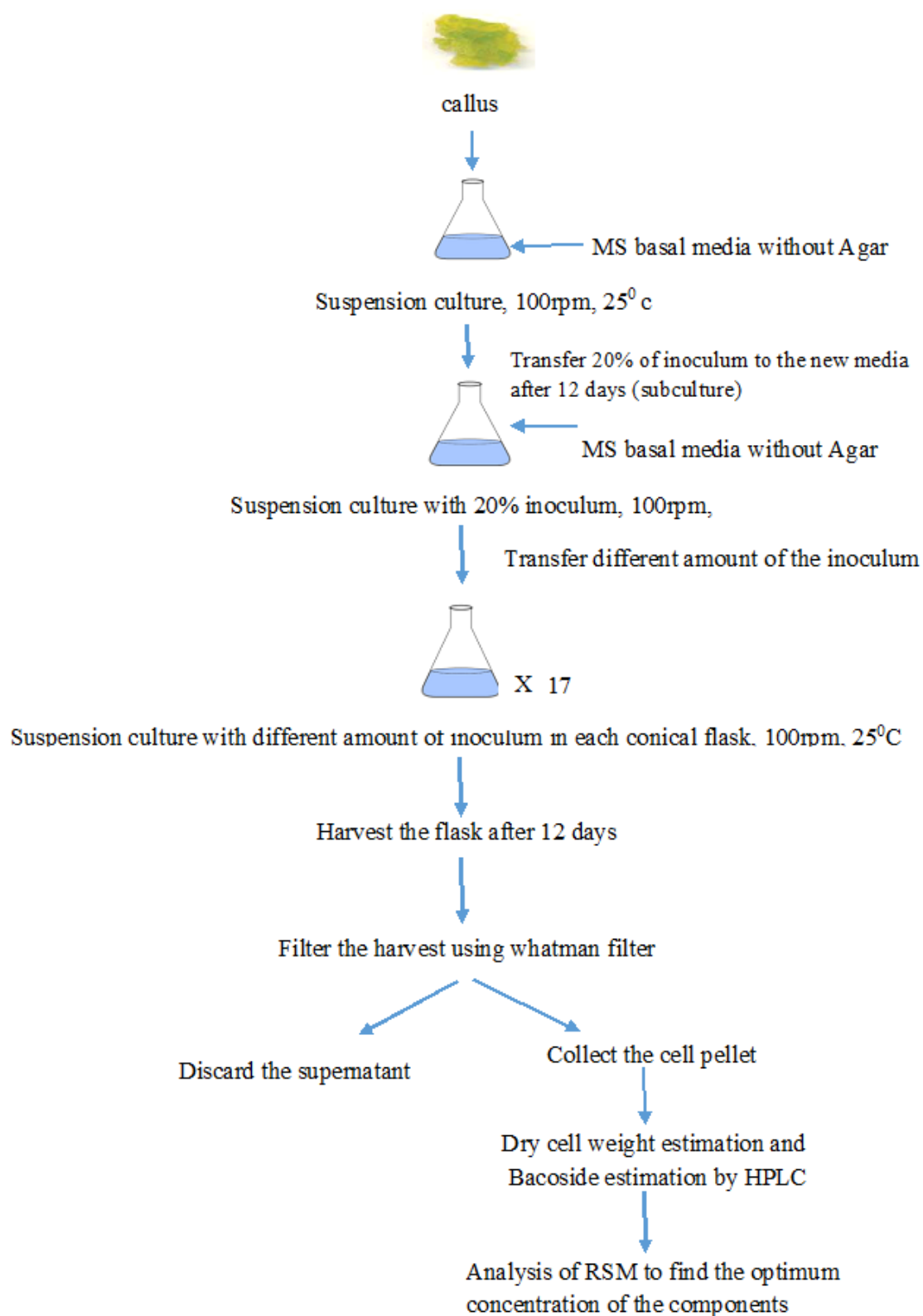
The significant factors with their high and low value has been shown in the table below (Table 4.1)

Table 4.1: Factors with high and low value used for CCD design

Factors	High value	Low value
Sucrose	60 g/L	30 g/L
Phosphate	2 mM	0.20 mM
Inoculum size	2 g/L FW	0.50 g/L FW

The experiments were done in suspension culture. For suspension culture 100 ml of media was prepared in 250 ml conical flask for each experiment and callus were inoculated to each conical flask according to the experimental data (Table 5.3). Then cultures were incubated in orbital shaker at 25 °C for 12 days at 100 rpm. After 12 days' dry cell weights of callus biomass was measured.

4.3.1 Flow diagram of RSM



4.4 Estimation of Bacoside by HPLC analysis

4.4.1 Metabolite extraction and sample preparation

Biomass from cell suspension cultures were separated at the end of the cultivation using filtration and dried at room temperature. Dry weights were measured to estimate the biomass production and the dried callus were used for the estimation of total Bacoside content. Extraction of Bacoside was performed as per the protocol described in literature (Deepak et al., 2005) with minor modifications as described below. Dried callus and crushed in a mortar pestle after adding 10 ml of methanol to it. Then the sample was sonicated at 24 kHz for 8 minutes. After sonication the sample was centrifuged at 7000 rpm for 10 minutes. Then the supernatant was collected and filtered through a 0.45-micron syringe filter. This filtrate supernatant (isolated sample) was used for HPLC analysis.

4.4.2 Standard preparation

Stock solution of standard Bacoside at a concentration of 1mg/ml was prepared. From stock solution five different concentration (30, 40, 50, 60, 70µg/ml) of standard Bacoside was prepared.

4.4.3 HPLC analysis

All the samples and standards were analysed by HPLC. Analytical separations were carried out by C18 guard column, using a gradient of Acetonitrile (A) and 0.05 % (v/v) Orthophosphoric acid (B) at a flow rate of 1.5ml/min as mobile phase. Column temperature was 30 °C. Detection was at 205 nm and the injected volume for each sample was 30 µl. Samples were run according to the elution programme (Table 4.2).

Table 4.2: Elution programme of HPLC

Run time (in minutes)	Acetonitrile (A)	0.05 % Orthophosphoric Acid (B)
0	30	70
25	40	60
35	60	40
40	60	40
45	30	70

Standard calibration curves were established by plotting the area of peaks against different concentration of standard Bacoside (Sigma, USA). Quantification of Bacoside in the samples was determined by using the regression equation of calibration curves.

4.5 Experimental validation of the model predicted optimized media

For the verification of the experimental model the optimum value predicted by the software were used to verified the media to determine the percentage of similarity. The predicted value was 3.65 g/L biomass yield when the amount of inoculum, concentration of sucrose and phosphate were 2 g/L DW, 30 g/L and 1.24 mM respectively.

Experiments were done in same method in suspension culture by taking the optimum concentration of the parameters obtained from the analysis. Suspension culture were done in three conical flasks. From two conical flasks the yield of Biomass was determined and the average was calculated for verification of the experimental model. Culture media from one conical flask was used for sampling to estimate the nutrient (sucrose, nitrate and phosphate) consumption.

4.6 Substrate consumption profile of *Bacopa monnieri* cell suspension culture

Medium from the suspension culture was collected under sterile conditions after every day for the analysis of sucrose, nitrate and phosphate. Cell biomass was estimated on dry cell weight basis.

4.6.1 Sucrose estimation

Sucrose was estimated by DNS method (Miller, 1959). Standard stock solution of sucrose at a concentration of 2 mg/ml was prepared. From the stock solution different concentration (0.2, 0.6, 1, 1.4, 1.8 mg/ml) of sucrose was prepared by diluting water. Then 0.25 ml 2N HCl was added to it for hydrolysis and immediately kept in boiling water bath for 10 minutes for the hydrolysis reaction to be taking place. Then it was cooled to room temperature and same amount of 0.25 ml 2N NaOH was added to neutralized the solution.

Then 1.5 ml of DNS reagent was added and kept in boiling water bath for 15 minutes for the reaction to be taking place. Then it was cooled in running tap water and finally 10 ml of distilled water was added to each of the solution. Blank was also prepared. Absorbance was measured in UV/V spectrophotometer at 540 nm. Sucrose estimation in sample was done in the same way. Concentration of sucrose in sample was determined with the help of regression equation of the standard curve.

DNS reagent preparation: i) 45 gm of sodium potassium tartrate was diluted in 75 ml of distilled water. ii) 1.5 gm of 3,5-dinitro salicylic acid was diluted in 30 ml of 2N NaOH. iii) both the solution was mixed and distilled water was added up to 150 ml.

4.5.2 Nitrate estimation

Nitrate was estimated by using salicylic acid method (Cataldo et al., 1975). Standard stock solution of 0.25 mg/L KNO_3 was prepared. From stock solution different concentration (0.0125, 0.025, 0.0375, 0.05, 0.0625 mg/ml) of standard solution was prepared by diluting water. Then 0.8 ml of Salicylic acid- H_2SO_4 reagent was added to each tube. After 20 minutes at room temperature 19 ml of 2N NaOH was added to raise the pH above 12. A blank was also prepared and the absorbance was measured by UV/V spectrophotometer at 410 nm. Sample was prepared in the same way. Concentration of nitrate in the sample was determined by the regression equation of the standard curve.

Salicylic acid- H_2SO_4 reagent was prepared by adding 5 % (w/v) salicylic acid in concentrate H_2SO_4 .

4.5.3 Phosphate estimation

Phosphate was estimated by Vandate-Molybdate reagent method (Kumar et al., 2007). Standard stock solution was prepared. For standard stock solution 0.2195 gm of KH_2PO_4 was diluted in distilled water up to 1 litre to get 50 mg/L phosphate concentration. Five standard (0.05, 0.1, 0.15, 0.2, 0.25 mg/ml) and one blank was prepared by taking different amount of stock solution in 50 ml conical flask. Then 10 ml of Vandate-Molybdate reagent was added to each standard. After 10 minutes distilled water was added to each conical flask up to 50 ml. absorbance was measured by UV/V spectrophotometer at 400nm.

Vandate-Molybdate reagent preparation: i) solution A: 2.5 gm of Ammonium Molybdate $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ was diluted in 30 ml distilled water. ii) solution B: 0.125 gm Ammonium Metavandate (NH_4VO_3) was diluted in 30 ml of boiling distilled water iii)

solution B was cooled to room temperature and 33 ml of concentration HCl was added. iv) Both the solution was mixed and distilled water was added up to 100 ml.

4.6 yield enhancement strategy for maximum bacoside production by elicitation

Methyl Jasmonate was used as elicitor to enhance the production of Bacoside. Optimized media was used for Bacoside production in suspension culture. One controlled was also run with the elicitation study. For elicitation study 5 mg/L Methyl Jasmonate was added and for controlled 100 μ L ethanol. Elicitor and control were added at day four. Elicitation study was done for a period of 7 days. Callus biomass was harvested after 7 days and dry cell weight was measured. Dry callus was used for Bacoside estimation by HPLC.

Chapter 5

Results and discussion

5.1 Root induction from shoot explant of *Bacopa monnieri*

After 4-5 days of inoculation root formation started from the shoot explants. It was found that MS medium supplemented with 1 mg/L IAA was effective for root induction



Fig 5.1: Rooting of the shoot explant of *B. monnieri*

5.2 Callus culture of leaf explants of *Bacopa monnieri*

Surface sterilization with 0.5 % Bavistin was effective. Plant growth regulators NAA at a concentration of 0.5 mg/L and BAP at a concentration of 0.1 mg/L were found to be efficient for callus induction. Callus was induced after 7-8 days of inoculation. Callus shown in the figure (Fig 5.2) was 14 days old.



Fig 5.2: Callus culture of leaf explant of *B. monnieri*

5.3 Estimation of Bacoside by HPLC

From HPLC analysis it was found that the area of peaks for the Bacoside was between the retention time 14 to 20. Five-point standard calibration curve was plotted between different concentration of standard Bacoside Vs areas. Amount of Bacoside was estimated with the help of regression equation obtained by the calibration curve of bacoside.

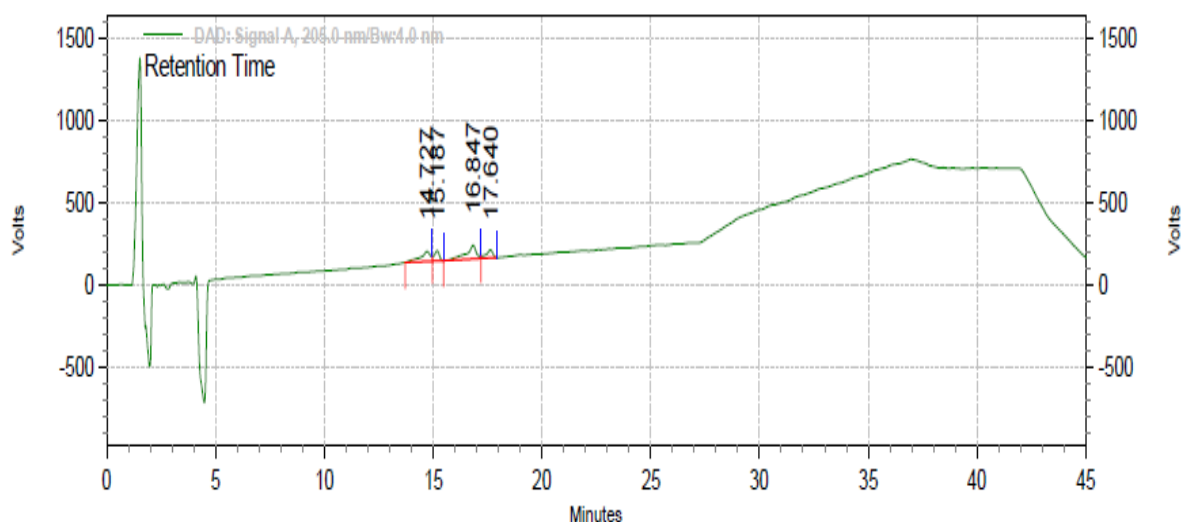


Fig 5.3: HPLC analysis of standard Bacoside

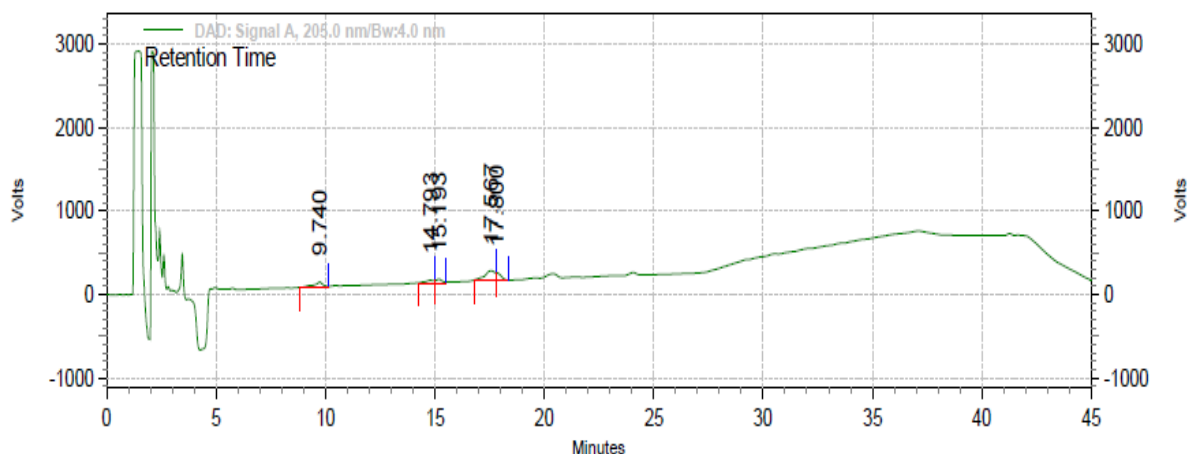


Fig 5.4: HPLC analysis of Bacoside in Test sample

5.3.1 Calibration curve of Bacoside

Standard calibration curve was plotted between the different concentration of standard Bacoside and the total areas against the corresponding peaks.

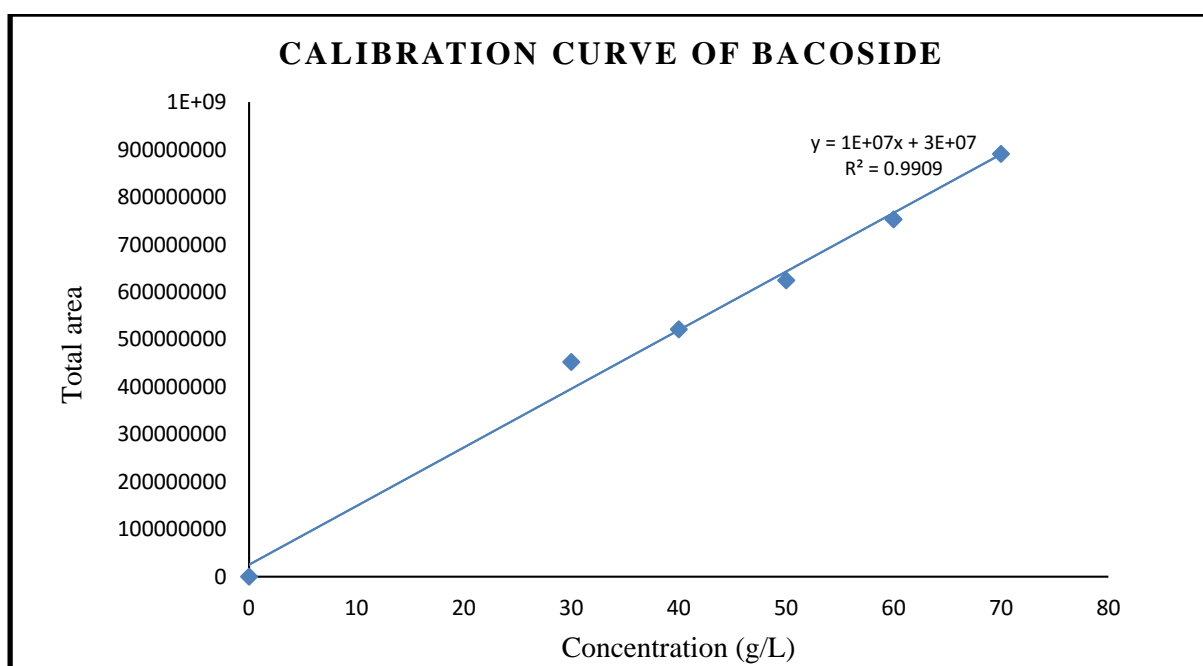


Fig 5.5: Calibration curve of Bacoside

5.3.2 Estimation of Bacoside

Bacoside in the test samples were estimated by using the formula attached in appendix 8.1.

5.4 Media optimization by RSM

For the dry cell weight (DCW) measurement, callus was dried in room temperature until a constant weight was achieved. Then the first response (DCW) were analysed with the help of software. Contour plot and 3D surface plot were studied to explore the relationship between the variables. Three -D surface plot is alternative to the contour plot. It gives more clear 3-dimensional view as compared to the contour plot. In the first case sucrose and phosphate were vary while inoculum (2 g/L) was kept constant and it was found that the maximum yield of DCW was 3.64 g/L (Fig 5.6, Fig 5.7). In the second case sucrose and inoculum were vary while phosphate (1.10 mM) was kept constant and it was found that the maximum yield of DCW was 3.62 g/L (Fig 5.8, Fig 5.9). In the third case inoculum and phosphate were vary while sucrose (30.77 g/L) was kept constant and it was found that the maximum yield of DCW was 3.61 g/L (Fig 5.10, Fig 5.11).

Second response (Bacoside) was also analysed by the software. Both contour and three dimensional plot were studied to explore the relationship between the variables. In the first case sucrose and phosphate were vary while inoculum (1.25 g/L) was kept constant and it was found that the maximum yield of bacoside was 0.39 mg/g DW (Fig 5.12, Fig 5.13). In the second case sucrose and inoculum were vary while phosphate (1.10 mM) was kept constant and it was found that the maximum yield of bacoside was 0.46 mg/g DW (Fig 5.14, Fig 5.15). In the third case inoculum and phosphate were vary while sucrose (45 g/L) was kept constant and it was found that the maximum yield of bacoside was 0.49 mg/g DW (Fig 5.16, Fig 5.17).

Then point prediction tables was generated which predicted the optimum value of the parameters. In case of biomass the maximum predicted yield was 3.65 g/L when the optimum concentration of sucrose, phosphate and inoculum was 30 g/L, 1.24 mM and 2 g/L respectively (Table 5.4). In case of bacoside maximum predicted yield was 0.49 mg/g DW when the optimum concentration of sucrose, phosphate and inoculum was 41.92 g/L, 0.22 mM and 1.98 g/L respectively (Table 5.5).

Model equation for both biomass and bacoside has been shown below.

Table 5.3: Table contains experimental data for RSM

Std	Run	Block	Factor A: Inoculum g/L DW	Factor B: Sucrose g/L	Factor C: Phosphate mM	Response DCW g/L	Response Bacoside mg/g DW
5	1	Block1	0.5	30	2	0.32	0.408
3	2	Block1	0.5	60	0.2	0.5	0.284
11	3	Block1	1.25	45	1.1	1.56	0.176
9	4	Block1	1.25	45	1.1	1.56	0.176
10	5	Block1	1.25	45	1.1	1.56	0.176
2	6	Block1	2	30	0.2	3.32	0.108
6	7	Block1	2	30	2	3.8	0.093
4	8	Block1	2	60	0.2	4.26	0.145
7	9	Block1	0.5	60	2	0.27	0.430
1	10	Block2	0.5	30	0.2	0.44	0.522
12	11	Block2	1.25	45	1.1	1.56	0.368
8	12	Block2	2	60	2	0.48	0.176
19	13	Block2	1.25	45	1.1	1.56	0.176
18	14	Block2	1.25	45	2.61	1.8	0.115
14	15	Block2	2.51	45	1.1	4.94	0.472
17	16	Block2	1.25	45	-0.41	0.31	0.411
20	17	Block2	1.25	45	1.1	1.56	0.176
16	18	Block2	1.25	70.23	1.1	1.71	0.112
13	19	Block2	-0.01	45	1.1	0	0
15	20	Block2	1.25	19.77	1.1	1.27	0.120

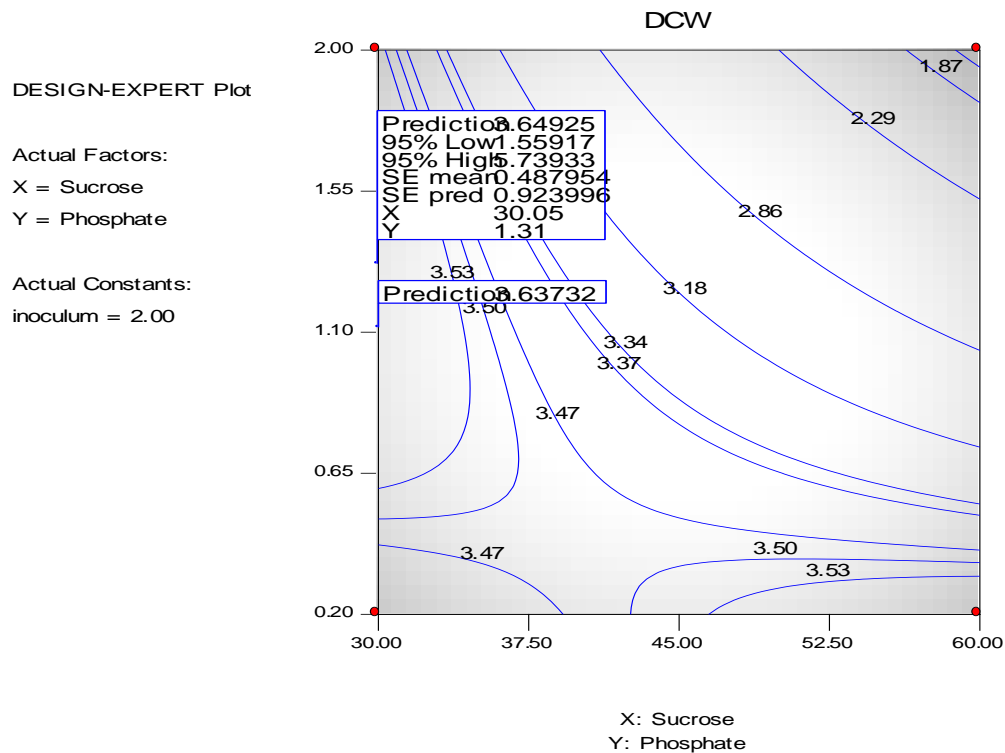


Fig 5.6: Contour plot showing interaction between sucrose and phosphate for DCW

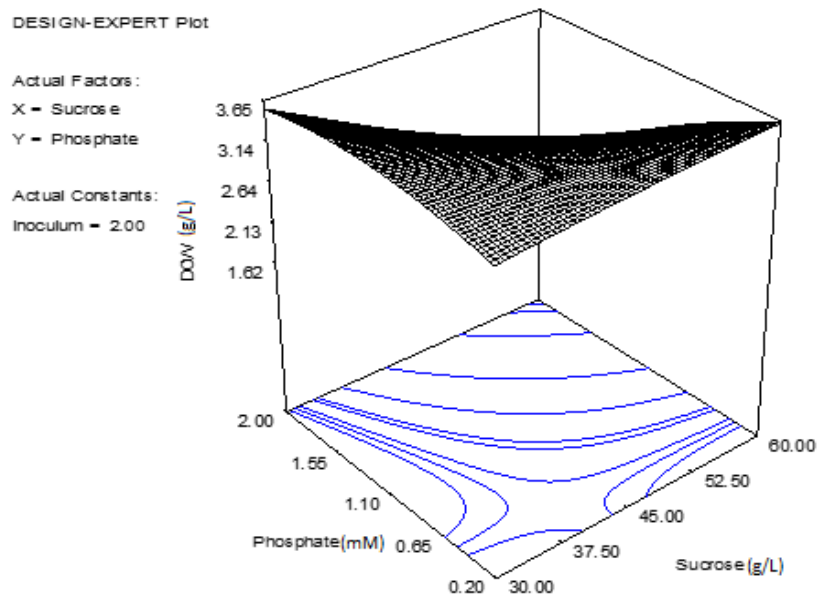
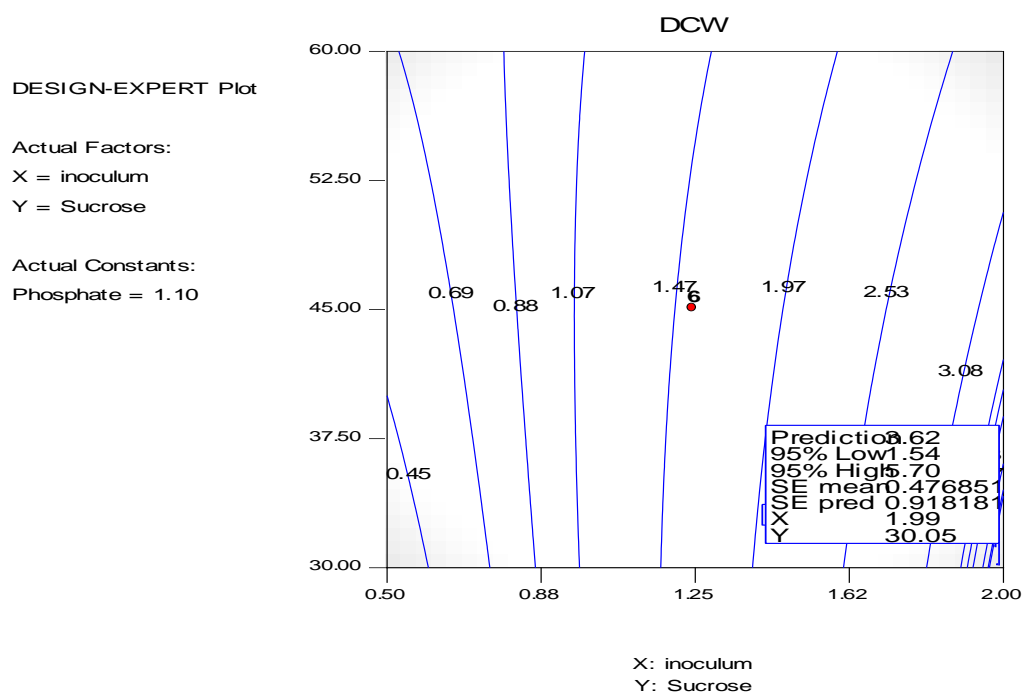
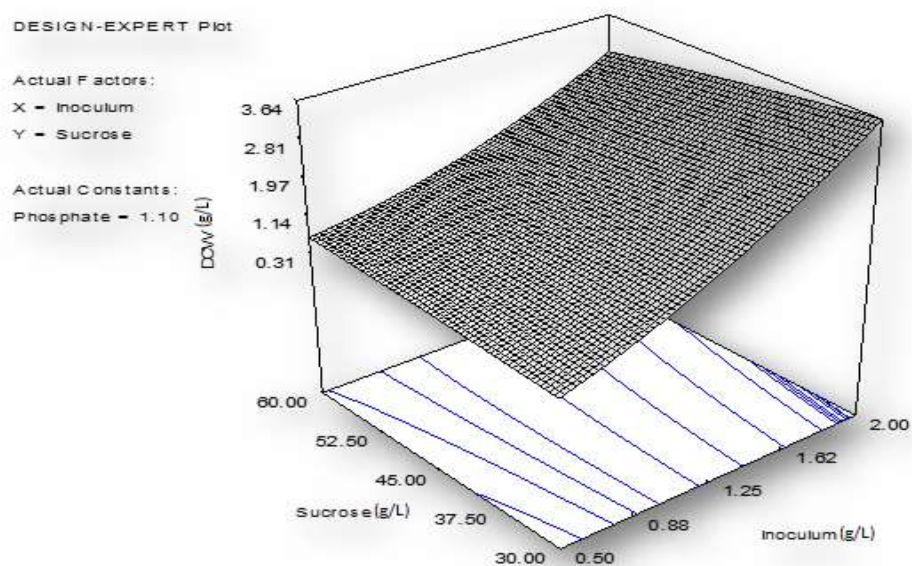


Fig 5.7: 3D surface plot showing interaction between phosphate and sucrose for DCW**Fig 5.8: Contour plot showing interaction between inoculum and sucrose for DCW****Fig 5.9: 3D surface plot showing interaction between sucrose and inoculum for DCW**

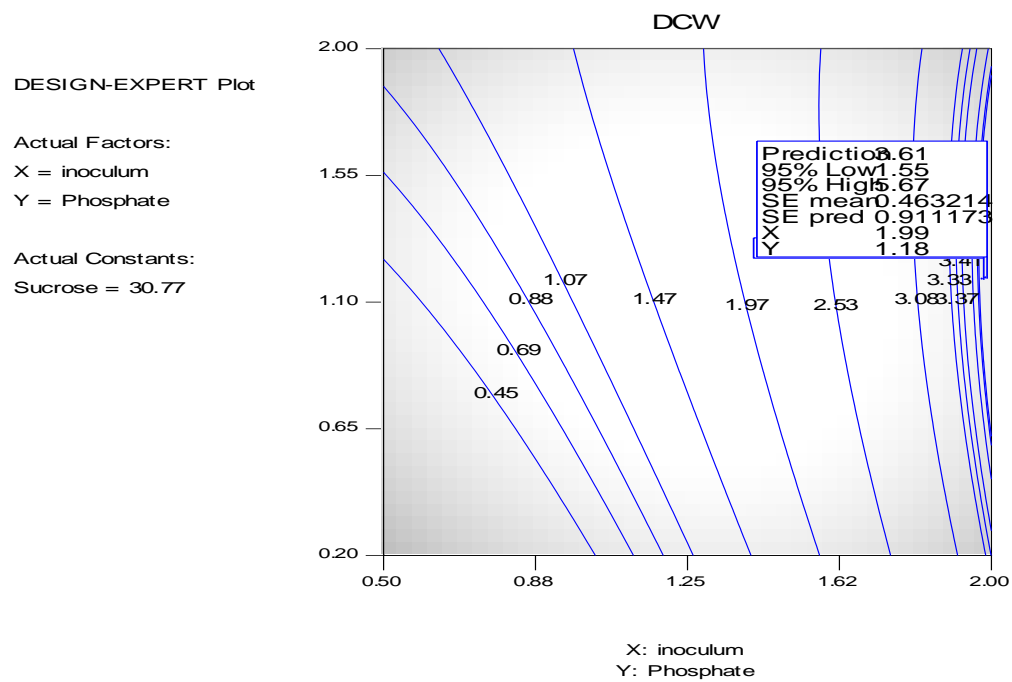


Fig 5.10: Contour plot showing interaction between inoculum and phosphate for DCW

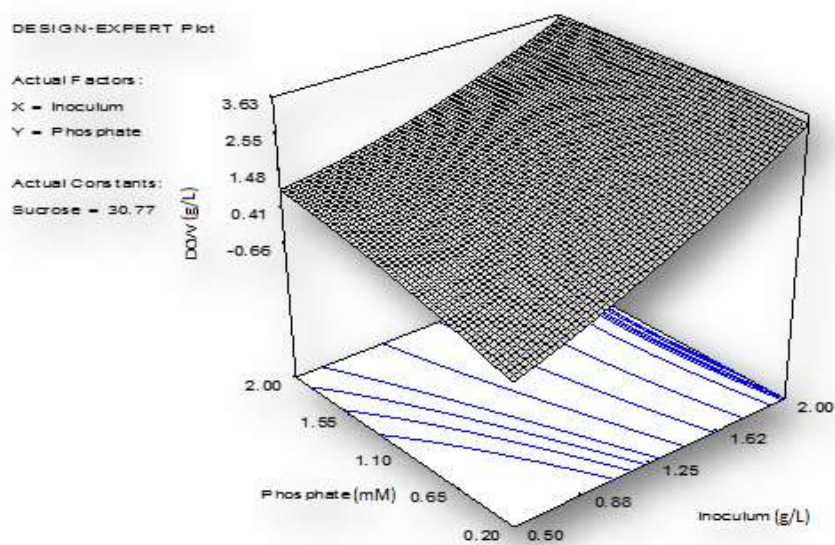


Fig 5.11: 3D surface plot showing interaction between inoculum and phosphate for DCW

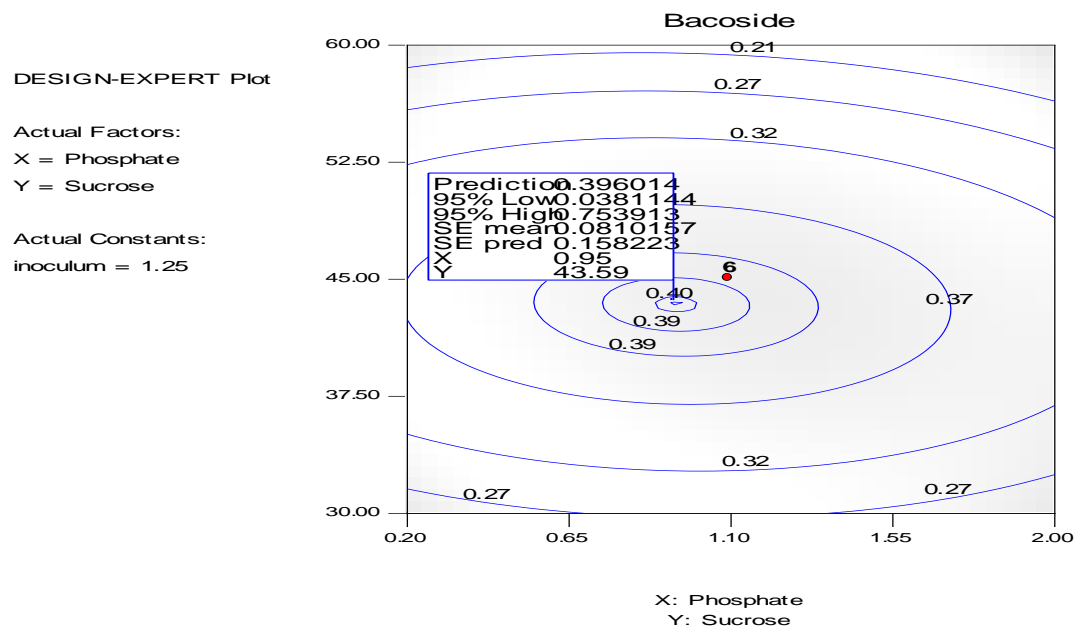


Fig 5.12: Contour plot showing interaction between phosphate and sucrose for bacside

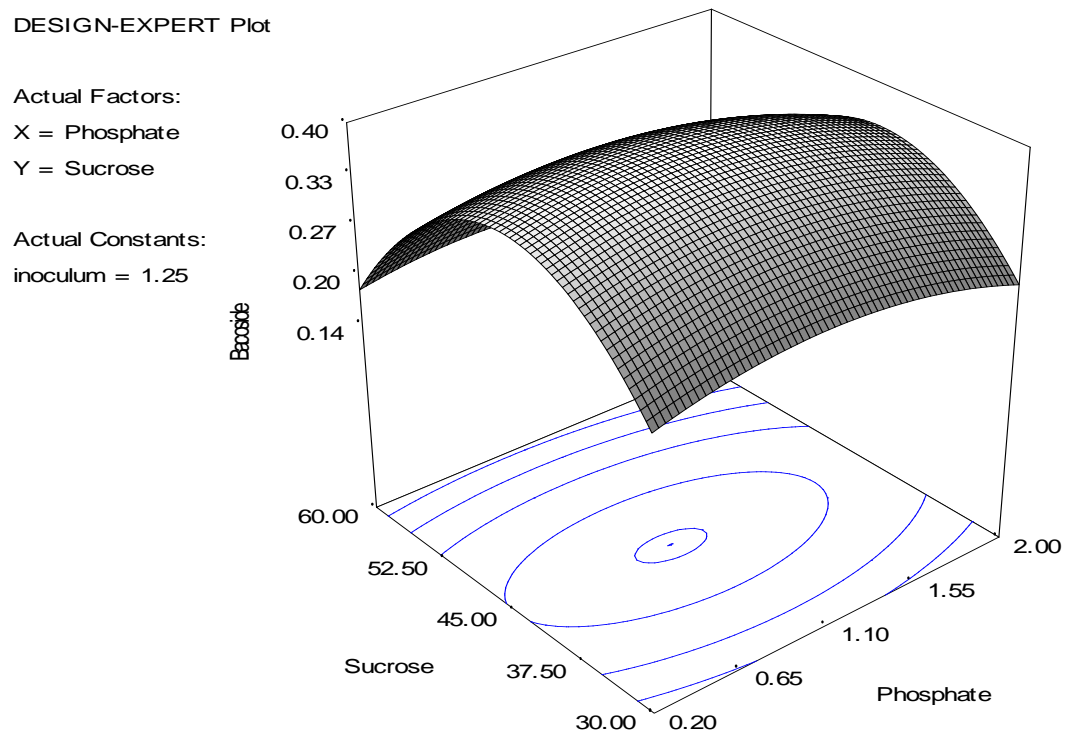


Fig 5.13: 3D surface plot showing interaction between phosphate and sucrose for bacside

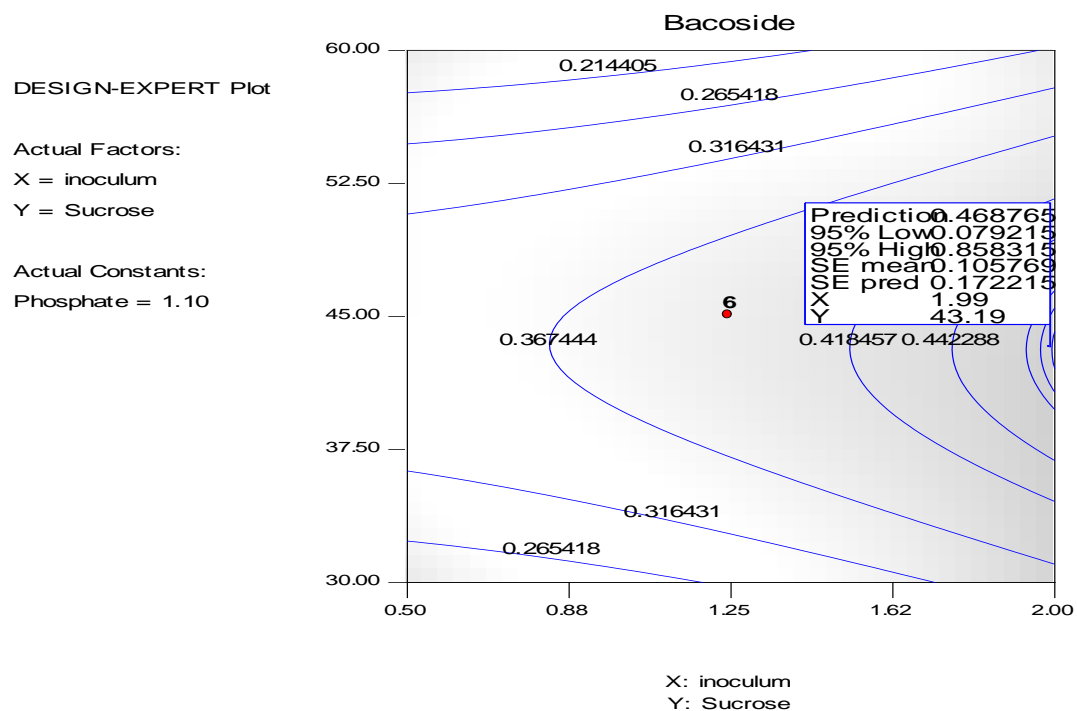


Fig 5.14: Contour plot showing interaction between inoculum and sucrose for bacoside

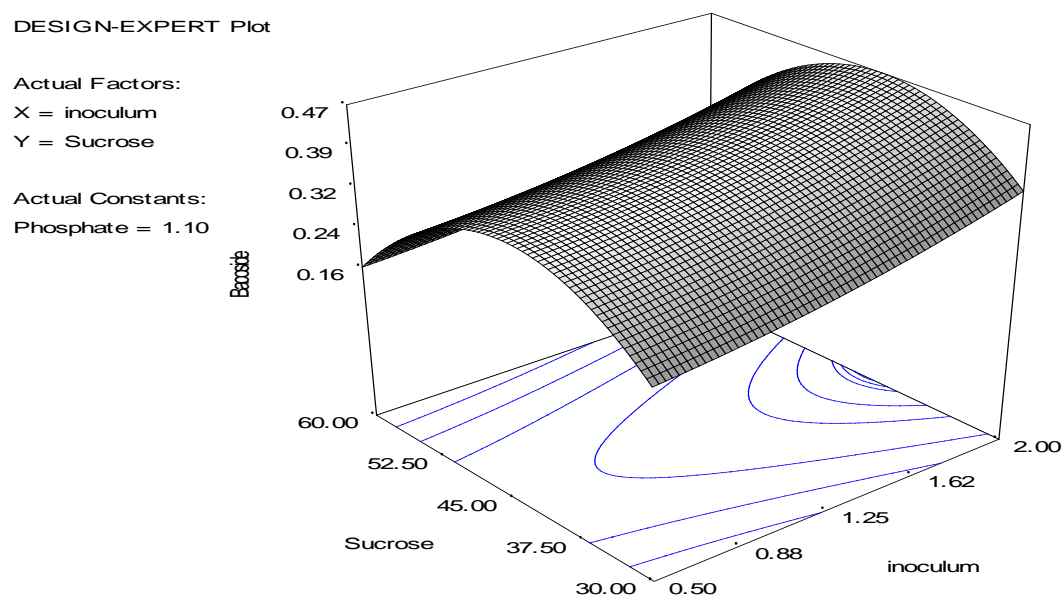


Fig 5.15: 3D surface plot showing interaction between inoculum and sucrose for bacoside

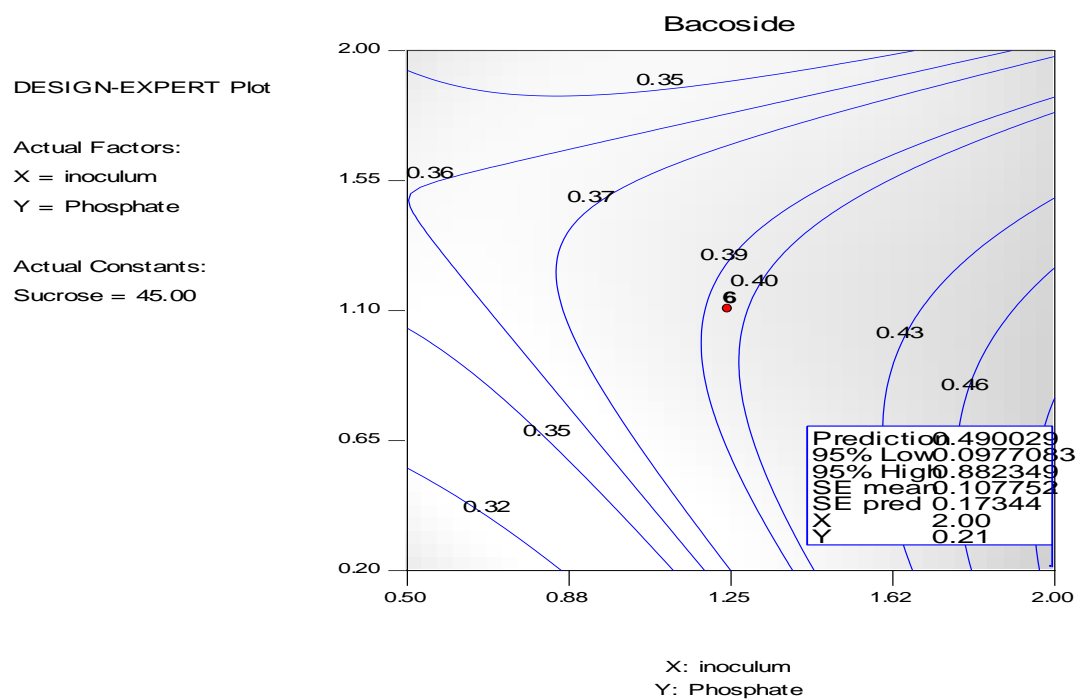


Fig 5.16: Contour plot showing interaction between inoculum and phosphate for bacside

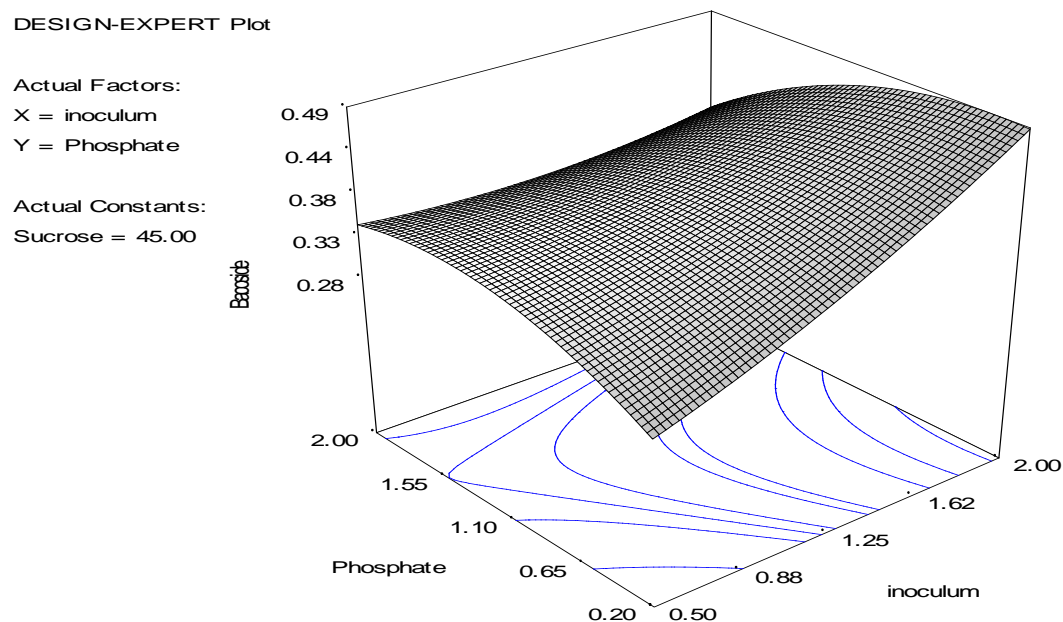


Fig 5.17: 3D surface plot showing interaction between inoculum and phosphate for bacside

5.4.1 Model equation for biomass and bacoside

$$DCW = 1.56 + 1.36A - 0.12B - 0.084C + 0.32A^2 - 0.026B^2 - 0.18C^2 - 0.30AB - 0.37AC - 0.55BC$$

$$Bacoside = 0.39 + 0.058A - 0.035B - 0.014C + 0.016A^2 - 0.16B^2 - 0.040C^2 - 4.481 \times 10^{-3}AB - 0.048AC - 8.231 \times 10^{-3}BC$$

Where, A = inoculum size (g/L)

B = sucrose concentration (g/L)

C = phosphate concentration (mM)

Table 5.4: Point prediction method for optimal DCW production

Factor	Name	Level	Low Level	High Level
A	Inoculum	2	0.5	2
B	Sucrose	30	30	60
C	Phosphate	1.24	0.2	2

	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
DCW	3.65	0.48	2.55	4.74	0.92	1.56	5.73

Table 5.5: Point prediction method for optimal bacoside production

Factor	Name	Level	Low Level	High Level
A	Inoculum	1.98	0.5	2
B	Sucrose	41.92	30	60
C	Phosphate	0.22	0.2	2

	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high
Bacoside	0.49	0.11	0.25	0.73	0.17	0.096	0.88

5.5 Experimental validation of the model predicted optimized media

Validation of the experimental model was performed. Correlation between experimental and model predicted value was 99 % for biomass and 94 % for bacoside production. There was 2.23-fold improvement for biomass and 3.63-fold improvement for bacoside production.

Table 5.6: Experimental validation of the model predicted optimized media

	Experimental value		Model predicted value
	Unoptimized media	Optimized media	
Biomass	2	3.62 g/L	3.65 g/L
Bacoside	0.232	0.32 g/L	0.34 g/L

5.6 Substrate consumption profile of *Bacopa monnieri* cell suspension culture

Sucrose, nitrate and phosphate consumption by the callus biomass was estimated in the test sample (Fig 5.18). It was observed that the nutrient consumption was very slow initially and was increased rapidly after third days of inoculation up to sixth day and then it was remaining constant at the end of the time period.

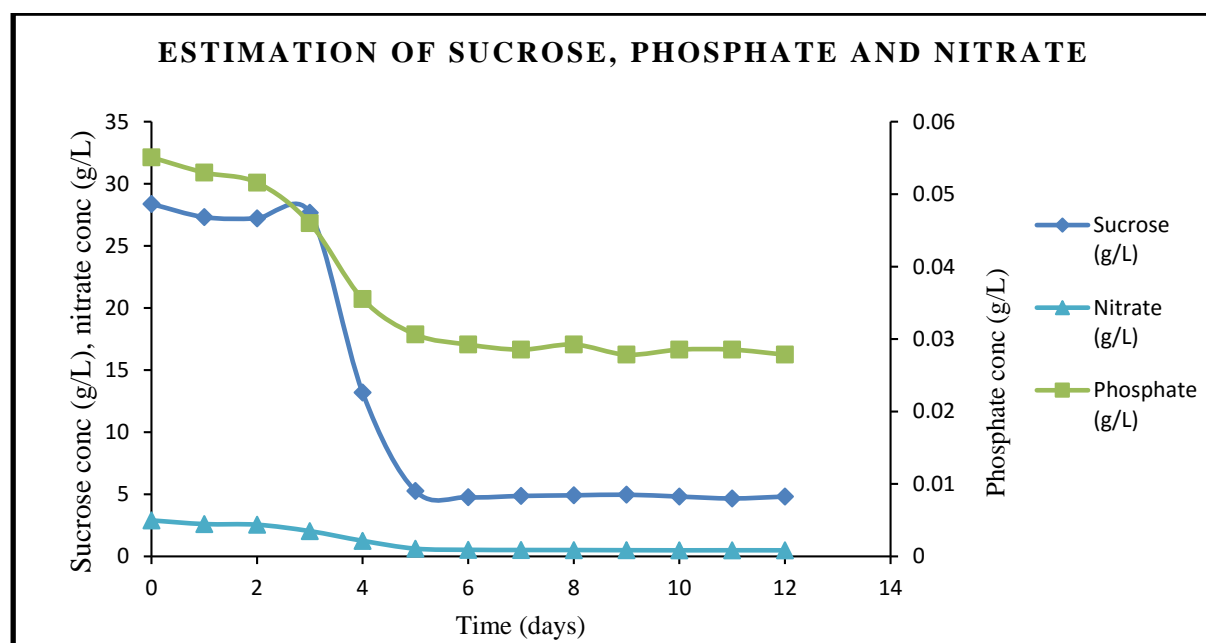


Fig 5.18: Substrate consumption profile of *Bacopa monnieri* cell suspension culture.

5.7 Yield enhancement strategy for maximum bacoside production by elicitation

Methyl Jasmonate (MJ) is widely present in higher plant and found to be play an important signalling role in the elicitation of plant defence response (Balbi and Devoto, 2008). Its exogenous application has been used to study their effects in plant metabolite production in medicinal plants (Gadzovska et al., 2007; Kirakosyan et al., 2006). Stock solution for Methyl Jasmonate was prepared in 95 % ethanol. Methyl Jasmonate at a concentration of 5 mg/L was used for the elicitation study and for controlled 100 μ L of 95 % ethanol was used. The increase in bacoside production in elicitation as compared to control was estimated. Bacoside produced by elicitation was 0.427 mg/g DW; a 1.4-fold increase as compared to control.

Table 5.7: Yield enhancement strategy by elicitation

Test samples	Biomass (g/L)	Bacoside (mg/g DW)
Without elicitor	3.62	0.32
With Elicitor	3.1	0.472

Chapter 6

Conclusion

The media was optimized using statistical media optimization tools and was 2 g/L for inoculum, 30 g/L for sucrose and 1.24 mM for phosphate. The correlation between experimental and model predicted value was 99 % for biomass and 94 % for bacoside production. By media optimization there was 2.23-fold improvement in biomass and 3.63-fold improvement in bacoside production. The yield of bacoside was enhanced by 1.4-fold by adding 5 mg/L Methyl Jasmonate in optimized media.

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Appendix

8.1 Estimation of Bacoside

Concentration of an bacoside in the test sample can be find out from the formula given below

$$C = \frac{A-I}{M}$$

Where, A= peak area of the bacoside in the test solution

I= y-intercept of the calibration curve

M= slope of the calibration curve

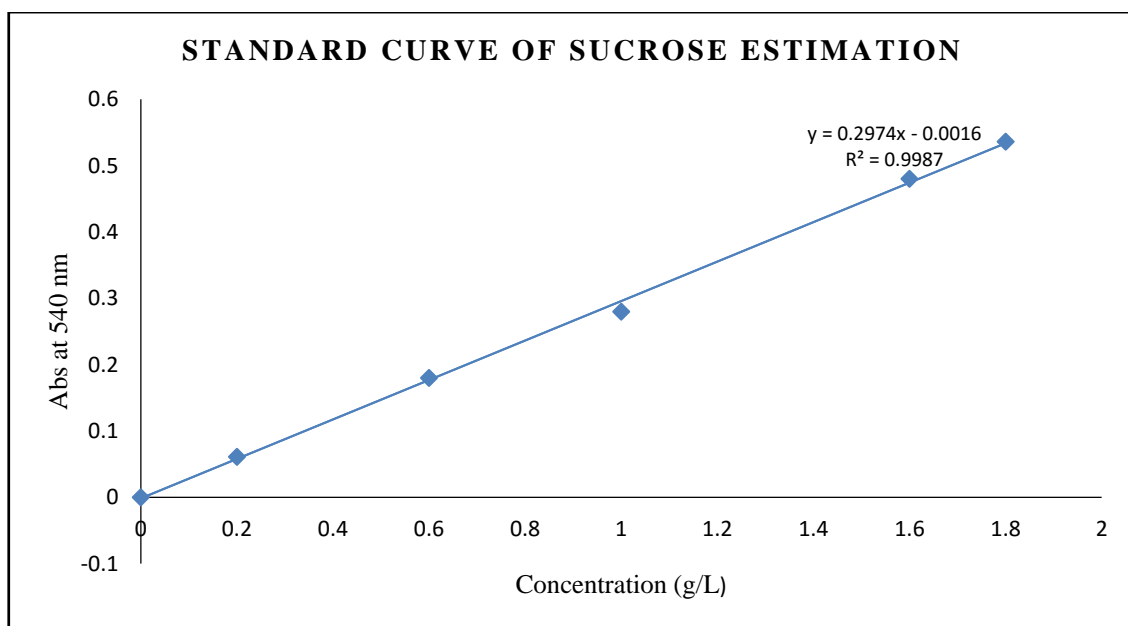
$$\text{Amount of bacoside in mg per g wet callus} = \frac{CV}{1000W}$$

Where, C= concentration of bacoside in test sample in mg/L

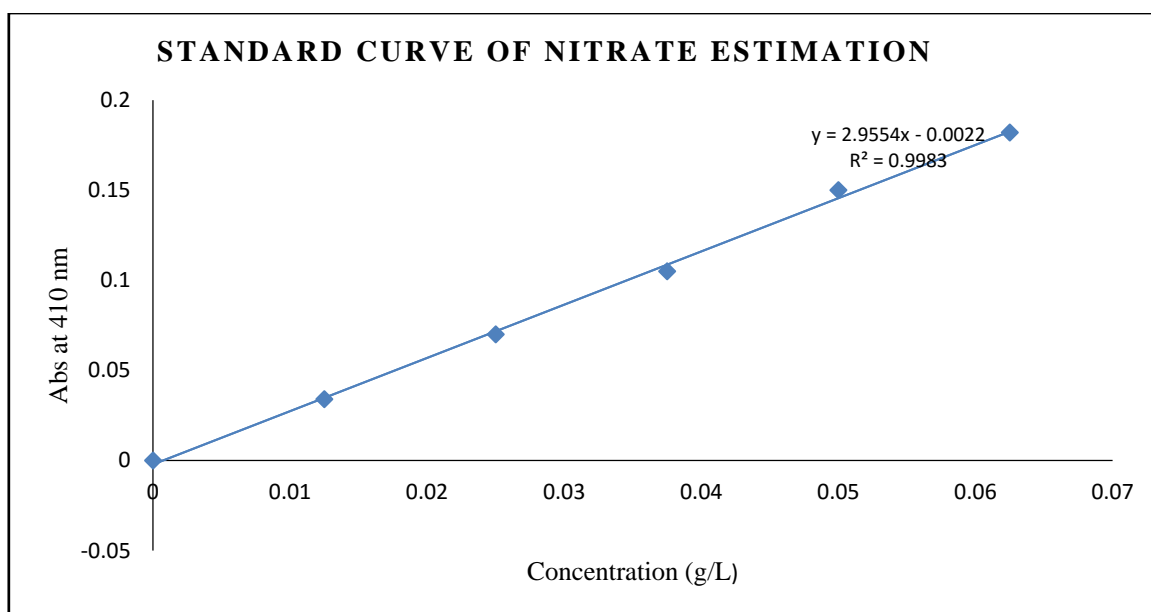
V= final make up volume in ml of test sample

W= weight, in g, of the sample used for the preparation of test sample

8.2 Standard curve of Sucrose estimation



8.3 Standard curve of Nitrate estimation



8.4 Standard curve of Phosphate estimation

